REGULATION OF CELLULAR ATP RELEASE

J. GREGORY FITZ, M.D.

DALLAS, TEXAS

ABSTRACT

Epithelial cells exhibit regulated release of ATP. Once outside of the cell, ATP in nanomolar concentrations functions as an autocrine/paracrine signal modulating a broad range of cell and organ functions through activation of purinergic receptors in the plasma membrane. The mechanisms responsible for ATP release have not been defined. In liver cells, there is evidence for ATP translocation through a conductive, channel-mediated pathway. In addition, indirect observations support a second potential mechanism involving exocytosis of ATP-enriched vesicles. Notably, stimuli that increase ATP release are associated with a five- to ten-fold increase in the rate of exocytosis; and inhibition of the exocytic response impairs cellular ATP release. More recent evidence suggests that these vesicles can be visualized, supporting the concept that in liver cells, ATP release is mediated in part by exocytosis of a pool of vesicles enriched in ATP, which can be mobilized within seconds in response to changing physiologic demands.

Purinergic Signaling—An Overview

ATP represents the primary molecule responsible for energy storage and transfer within the cell. Consequently, it was a surprise when Geoffrey Burnstock and coworkers proposed over 30 years ago that ATP was released from certain presynaptic neural cells and functioned as a neurotransmitter (reviewed in reference 1). Since that original description, ATP release has been identified in many cells types, and ATP and its metabolites have been shown to be potent regulatory molecules modulating a broad range of cell and organ functions. This paradigm is not limited to excitable cells. Rather, purinergic signaling pathways are now well described in many epithelial cells as well, and studies in liver, biliary, airway and many other models have demonstrated ATP to be a potent autocrine/paracrine signal (2–4).

In neuronal models, ATP is packaged within vesicles near the presynaptic membrane, and stimulation of exocytosis leads to release of

From UT Southwestern Medical Center, Dallas, TX 75390.

Address correspondence to: Greg Fitz, M.D., 5323 Harry Hines Boulevard, Dallas, TX 75390-9030. Telephone 214-648-3486; FAX 214-648-3088; Email greg.fitz@utsouthwestern.edu

ATP into the synaptic space, thus functioning as a classical neurotransmitter (1). In epithelia, the essential elements of the purinergic signaling cascade have been harder to define, but the molecular definition of many of the proteins involved has led to a general model illustrated in Figure 1. Under basal conditions, ATP is present intracellularly in concentrations of 3–5 mM. Signaling is initiated by nucleotide and/or nucleoside release, leading to a localized increase in extracellular ATP concentrations. Once outside the cell, ATP has a half-life measured in seconds as a result of a complex array of potent nucleotidases and other hydrolytic activities, which degrade ATP and generate ADP, AMP and adenosine (5). The actions of ATP itself, however, likely are limited to a narrow paracrine radius of a few hundred microns due to the rapid kinetics of these reactions and its dispersion by regional blood or fluid flow. Once outside the cell, ATP mediates its diverse effects by binding to and activating a broad range of receptors. Typically, the concentration of ATP required for half-maximal activation of purinergic receptors is 3–500 nM, values 1,000-fold lower than those inside the cell. Consequently, ATP released in quantities sufficient to initiate signaling does not appear to alter intracellular energy stores.

Many of these receptors have been defined molecularly, and include a) 4 separate P1 adenosine-preferring receptors, which are G-protein coupled and positively or negatively modulate adenylyl cyclase and

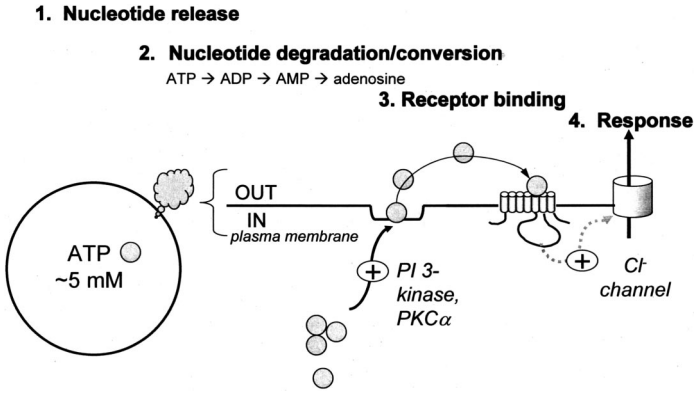

FIG. 1. Overview of epithelial purinergic signaling cascade. Extracellular ATP functions as a potent autocrine/paracrine signal in epithelia. The basic elements include 1) regulated release of ATP (and UTP) through ATP-permeable channels and/or exocytosis of ATP-containing vesicles, under active investigation, 2) rapid hydrolysis by ectonucleotidases to degrade ATP and generate metabolites which themselves have agonist properties, 3) binding to a broad range of P1, P2X and P2Y receptors in the plasma membrane, and 4) related signaling events according to the specific receptor involved.

other signaling pathways; b) 7 separate P2X (ionotrophic) receptors, which are receptor-gated ion channels. ATP binding to P2X receptors leads to a conformational change and a transmembrane pore is revealed resulting influx of Na^+ and likely Ca^{2+} from the extracellular space and depolarization of the membrane; and c) at least 8 separate P2Y (metabotrophic) receptors, which are also G-protein coupled receptors typically coupled to phospholipase C. There is a broad profile of agonist specificities amongst the P2Y family of receptors for ATP, ADP, and UTP depending on the receptor under investigation. These 19 receptors, and the number is growing, account for the diverse effector responses in target cells and mediate the physiologic effects of ATP. This local regulatory network is complex, with many control points. Moreover, target cells may express multiple types of P2X and P2Y receptors, and there is good evidence supporting release and even rapid interconversion of many types of nucleotides and nucleosides outside the cell. Thus, purinergic signaling is both rapid and versatile, and there is considerable variety amongst tissue types in the pathways involved (2).

Cell Volume Regulation by Extracellular ATP: Coupling Cell Transport to Metabolism

In the liver, activation of purinergic receptors by increasing concentrations of extracellular ATP leads to cell shrinkage, stimulation of glucose release, and protein catabolism (6,7). These findings are intriguing since liver cell volume is a dynamic parameter that undergoes physiologic changes between the fed and fasted states, coupling cellular transport to many organ-level functions (6). These and other considerations have led to the concept that changes in cell volume *per se* represent a signal regulating liver function. Specifically, increases in cell volume trigger a signaling cascade with potent anabolic effects, whereas decreases in cell volume have opposite effects (6,8). The possibility that extracellular ATP might serve as an autocrine signal involved in liver cell shrinkage (or recovery from cell swelling) has been evaluated (9). The principal findings that i) increases in cell volume stimulate ATP release, ii) removal of extracellular ATP or blockade or P2 receptors eliminates cell volume recovery from swelling, and iii) exposure to exogenous ATP activates ion channels involved in cell volume regulation support a pivotal role for ATP in cell volume recovery in a manner analogous to that shown in Figure 1. Thus, one role of ATP release in the liver involves regulation of cell volume. A

similar role for ATP release has been identified in other cell types, but it is not universal.

Demonstration of Hepatic ATP Release by Luminometry

An example of liver cell release of ATP in response to increases in cell volume is shown in Figure 2. In this example, a cover slip containing cells was mounted in a luminometer, and luciferin-luciferase was added to the extracellular bathing solution as described by Schwiebert and colleagues (10). With this assay, the appearance of ATP outside of the cell results in photon release as measured by arbitrary light units (ALU). Under resting conditions, extracellular ATP is low; and exposure to isotonic buffer to control for mechanical stimulation or cell injury has a small effect. Exposure to hypotonic buffer to stimulate an increase in cell volume causes a rapid increase in ALUs, consistent with volume-stimulated ATP release. Analogous responses have been detected in liver and biliary cell lines, airway and a variety of other cell models (10–12). These observations in cultured cells likely are relevant *in vivo* since in intact liver, ATP is detectable in hepatic veins draining the liver, and in bile, where it stimulates Cl^- secretion and

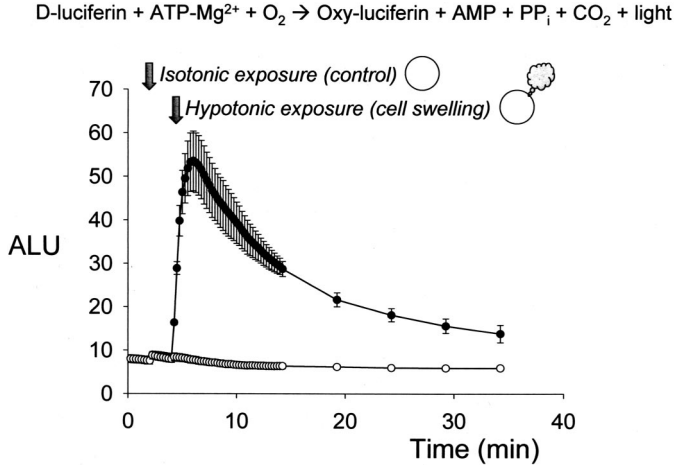

FIG. 2. Detection of extracellular ATP by the luciferin-luciferase reaction. Schwiebert *et al* (10) developed an assay based on luciferin-luciferase wherein increasing concentrations of ATP are detected as an increase in photon generation in Arbitrary Light Units (ALU). In the example shown, coverslips containing cells were mounted in a luminometer with luciferin-luciferase in the bathing solution as described (11,23). Addition of isotonic media control for mechanical stimulation had small effects. Addition of an equal volume of hypotonic media to cause cell volume increase was associated with an increase in ALU, consistent with volume-stimulated ATP release.

bile formation (13,14). ATP release in these models is not associated with cell injury, or detectable loss of cytosolic ATP content. However, the molecular mechanisms for movement of ATP from inside the cell to the extracellular space have not been defined.

Mechanisms of Cellular ATP Release

Two general mechanisms for ATP release by epithelia have been proposed, including conductive release through ion channels, and exocytic release of ATP-enriched vesicles. These are illustrated in Figure 3 and are not mutually exclusive since exocytosis could in addition lead to insertion into the plasma membrane of ATP-permeable ion channels. A role for ion channels in ATP translocation is generally accepted and seems logical since ATP at physiological pH values is largely anionic (e.g., $MgATP^{2-}$), and since there is a large concentration gradient favoring movement of ATP out of the cell. Accordingly, opening of a conductive pathway would lead to a localized increase in $MgATP^{2-}$ outside of the cell. Further, voltage-dependent anion channels (VDACs) are known to translocate ATP across the mitochondrial membrane. In liver cells, exposure to hypotonic buffer increases ATP conductance ~30-fold, and expression of the P-glycoprotein product of multidrug resistance (*mdr*) genes increases ATP release (9,11). However, *mdr* itself does not seem to be the anion channel involved. In airway cells, expression of a plasmalemmal splice variant of VDAC (VDAC-1) is associated with higher rates of ATP release, and cells from VDAC-1 knockout mice show impaired ATP release and cell volume

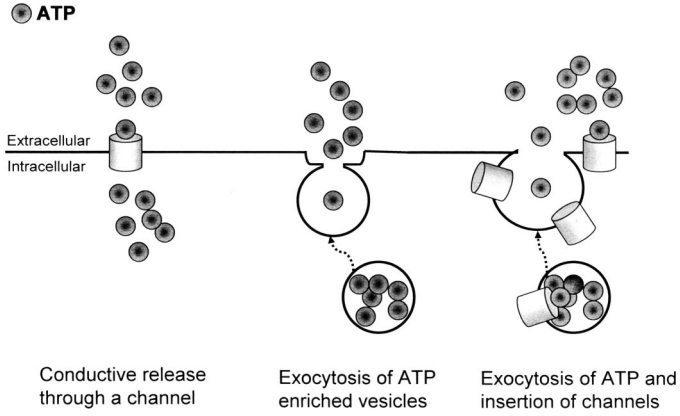

FIG. 3. Theoretical mechanisms of cellular ATP release include opening of ATP-permeable ion channels; exocytosis of vesicles enriched in ATP; and/or exocytosis of ion channel-containing vesicles.

regulation (12). Further, there is direct evidence of a role for ATP release through analogous large conductance channels in macula densa cells (15). Since similar channels are present in the plasma membrane of liver cells (unpublished observation), it seems likely that they serve a similar role. However, this assumption has not been tested, and several different anion channel types appear permeable to ATP under specific conditions.

Is there, in addition, a role for exocytosis? There is increasing evidence that such is the case. In pancreatic acinar cells, quinacrine staining of cellular ATP stores reveals a punctuated distribution of fluorescence consistent with vesicles enriched in ATP (16). Several lines of evidence support a similar mechanism in hepatocyte and cholangiocyte cell lines, representing the two primary epithelial cell types in the liver. First, increases in cell volume stimulate phosphatidylinositol (PI) 3-kinase, and the resulting increase in 3-phosphorylated lipids are known to regulate vesicular exocytosis (17,18). Second, inhibition of PI 3-kinase by wortmannin or LY294002 blocks volume-stimulated ATP release, and prevents cell volume recovery from swelling (19). Third, intracellular dialysis with an antibody to the p110 catalytic subunit of PI 3-kinase also prevents these events; whereas intracellular delivery of phosphatidylinositol 3,4-bisphosphate activates volume-regulatory signaling (19,20).

The concept that vesicular exocytosis contributes to volume-sensitive ATP release has been evaluated more directly in a biliary cell model (21). The fluorescent probe FM1-43 was utilized to measure the rate of exocytosis in single cells (Figure 4); and extracellular ATP was assessed by the luciferin-luciferase method (Figure 5). Interestingly, increases in cell volume stimulated parallel changes in both exocytosis and ATP release through a mechanism dependent on both protein kinase C and PI 3-kinase. Using the FM1-43 method, total cellular fluorescence provides a measure of the amount of membrane exposed to the dye. Consequently, exocytosis and insertion of new vesicular membrane leads to an increase in cellular fluorescence. For example, increases in cell volume (30% hypotonic exposure) caused a 10-fold increase in ALU and a 15% increase in fluorescence. These and other observations suggest that increases in cell volume result in a rapid increase in exocytosis sufficient to replace 15–30% of the plasma membrane within minutes. This value corresponds to recruitment of ~10,000 or more vesicles in a single cell; and inhibition of this exocytic response by blockade of PI 3-kinase or protein kinase C substantially inhibits volume-dependent ATP release (21).

Thus, while vesicular exocytosis is necessary for volume-sensitive

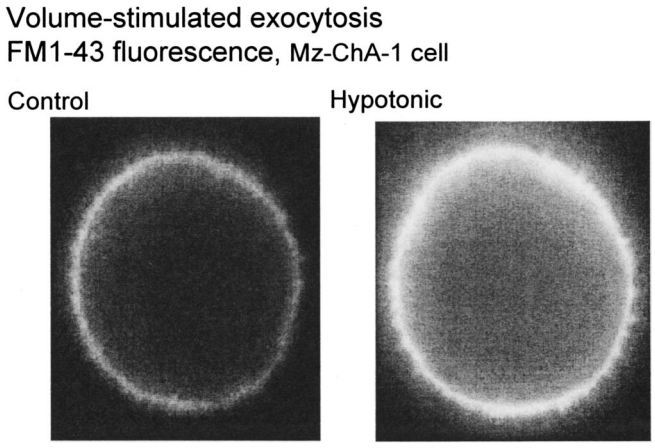

FIG. 4. Detection of exocytosis through FM1-43 fluorescence. FM1-43 fluorescence provides a measure in real time of exocytosis as new membrane-containing vesicles come in contact with FM1-43 in the media (24). In this model biliary cell, images were obtained before and ~2 min after exposure to hypotonic media (−30%) to increase cell volume. The increase in plasma membrane fluorescence occurred as a result of vesicular exocytosis. Reproduced from Am. J. Physiol. 286:G538–546, 2004 with permission.

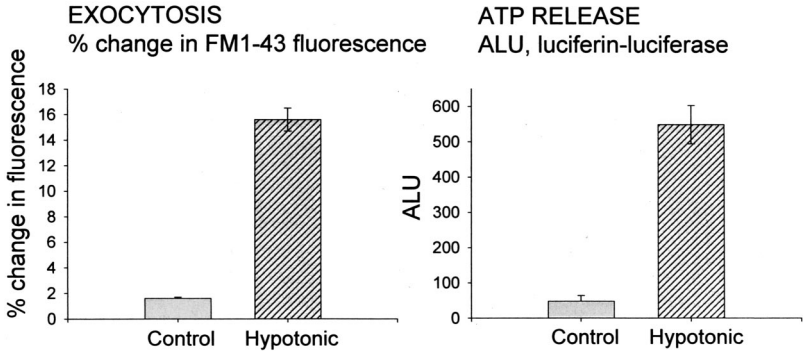

FIG. 5. Effect of cell volume increases on exocytosis and ATP release. In model biliary cells as described in Figure 4, exposure to hypotonic media to increase cell volume caused within minutes an increase in exocytosis to values sufficient to replace ~15% of the plasma membrane min^{-1} as assessed by FM1-43 fluorescence; and caused a parallel increase in ATP release. Inhibition of exocytosis by blockade of protein kinase C or PI 3-kinase causes a parallel inhibition, suggesting that exocytosis and ATP release are linked. Data redrawn from Am. J. Physiol. 286:G538–546, 2004.

ATP release, the big question of what is in these vesicles remains unanswered. In preliminary studies, examination of the location of intracellular ATP stores in liver and biliary cells using quinacrine shows a punctuated pattern of distribution, which might suggest that

there are releasable vesicles in which ATP is concentrated (A.P. Feranchak, unpublished observations). This mechanism would be similar to observations in endothelial and pancreatic acinar cells (16,22). In acinar cells, cholinergic stimulation leads to release of vesicular contents and ATP concentrations as high as 9 μM immediately adjacent to the cell surface (16). Alternatively, there could in addition be ATP-permeable channels in vesicular membranes, where exocytosis would lead to a larger number of channels in the plasma membrane and an increase in membrane ATP permeability. The direct evidence for or against such a model awaits specific information about the molecular identity of the channel type (or more likely types) involved.

Summary and Conclusions

These and other observations support the concept that ATP release from liver cells (hepatocytes and cholangiocytes) depends in part on exocytic release of a distinct population of vesicles enriched in ATP and/or ATP permeable ion channels. The rapid insertion of up to 10,000 vesicles per cell is sufficient to replace 10–30% of the entire plasma membrane within a few minutes. Accordingly, this mechanism permits rapid changes in the functional properties of the plasma membrane to meet rapidly changing physiological demands. Moreover, definition of the mechanisms involved might provide attractive targets for pharmacologic modulation of diverse liver function known to be regulated by ATP, including protein synthesis, glucose metabolism and bile formation.

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DISCUSSION

Mackowiak, Baltimore: What does quinacrine do to ATP production and release?

Fitz, Dallas: It doesn't seem to do much. It's a promiscuous dye that is incorporated into a large number of acidic vesicles, and in the neurobiology literature, has been used to localize ATP into larger vesicles. It has been shown to be selectively concentrated, and because it is fluorescent, it is readily visualized and serves as a good marker. There have not been other good markers for vesicular ATP on the market, so it is imperative to look at quincrine fluorescence with ATP release in other assays.

Luke, Cincinnati: Is this ATP release, for example, part of the brain's response to hypotonic hyponatremia? Because there are a lot of risk factors for both the hyponatremic encephalopathy and for the demyelination syndromes; some of these are related to hypoxia and inability of brain cells to defend against hypo-osmolality.

Fitz: Well ATP is released for sure. In nerve cells, both centrally and peripherally, ATP is one of the non-adrenergic non-cholinergic transmitters. The response, however, is complex due in part to the diversity of receptors. The seven P2X receptors are receptor-gated cation channels, where sodium and calcium influx are depolarizing; while the P1 and P2Y receptors are G protein-coupled receptors. The result is significant spatial geometry issues with different responses depending on where and what type of receptors are expressed.

Gotto, New York: Since triglyceride accumulation in the liver appears to have some role in the fibrotic reaction, what are your thoughts about the relationship between this ATP loss and triglyceride accumulation?

Fitz: This is an important question, and it relates almost certainly to the epidemic of fatty liver. Fatty liver for a long time was thought to be relatively benign. It is now the number 2 cause of liver transplantation behind hepatitis C. While I do not know the true clinical significance of fatty liver and ATP signaling, it is clear that if you induce fatty acid accumulation in isolated liver cells—by exposure to amiodarone, for example, to inhibit mitochondrial beta oxidation of fatty acids—the increase in fatty acid accumulation inhibits exocytosis and inhibits the physiologic release of ATP. While I do not know the physiological implications, this inhibition is a very consistent finding.

Gotto: Thank you.

Alexander, Atlanta: That was elegant, Greg. I really appreciated such a fantastic presentation. I was intrigued with the model slide that you showed apparent coupling of integrin receptor signaling and with flow-mediated signaling. Two questions: Is that one of the primary activation mechanisms you hypothesized? And the second, there are many studies showing recycling of various signaling receptors pathways that involve vesicular trafficking. Is the model you presented generalizable to many of these other recycling mechanisms?

Fitz: We think this model will prove to be more general. We also think that integrin signaling will prove to contribute to flow- or shear stress-induced ATP release based on preliminary studies and results by Erik Schwiebert at Alabama. A picture is emerging wherein integrin linked tyrosine kinase signaling is a contributor to these trafficking events and ATP release. There are a number of diseases, ADPKD for example, that seem to be associated with defective ATP release. So whether replacement or reconstitution of these mechanisms might be beneficial, I do not know.